

## **Exercise training restores uncoupling protein-3 content in limb muscles of patients with chronic obstructive pulmonary disease**

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## ABSTRACT

Oxidative capacity and uncoupling protein-3 (UCP3) content are reduced in limb muscles of patients with chronic obstructive pulmonary disease (COPD). It has been hypothesized that the physiological role of UCP3 is to protect mitochondria against lipotoxicity in case where fatty acid influx exceeds the capacity to oxidize them. Exercise training improves oxidative capacity and reduces UCP3 protein content in healthy subjects, but the response of UCP3 to training in COPD is unknown. We studied the effect of exercise training on UCP3 content in limb muscles of COPD patients. For this, seven healthy age-matched subjects and thirteen patients with COPD were studied. All patients were admitted to an eight-week exercise training intervention. Exercise capacity was assessed by means of an incremental cycle ergometry test. Biopsies were taken from the vastus lateralis in which UCP3 and lipid peroxidation levels were determined by Western Blotting. Citrate synthase and 3-hydroxyacyl-CoA dehydrogenase (HAD; an enzyme involved in fatty acid oxidation) were measured as indices of muscle oxidative capacity. UCP3 in COPD was ~50% lower compared to healthy age-matched controls. In COPD, training induced upregulation of UCP3 (from  $67.7 \pm 41.8$  to  $113.8 \pm 104.2$  AU,  $p=0.062$ ), especially in the patients who showed no increase in HAD activity (from  $80.9 \pm 52.6$  to  $167.9 \pm 109.1$  AU,  $p=0.028$ ), whereas lipid peroxidation levels remained unaltered. We conclude that exercise-training can restore muscle UCP3 protein level in COPD and the nature of this response complies with the hypothesis that UCP3 may protect against lipotoxicity.

Keywords: skeletal muscle, obstructive lung diseases, exercise therapy, 3-Hydroxyacyl CoA Dehydrogenases, mitochondrial uncoupling protein 3

## INTRODUCTION

Evidence is accumulating that peripheral skeletal muscle impairment significantly contributes to exercise intolerance in patients with chronic obstructive pulmonary disease (COPD). This muscular dysfunction is reflected by reduced muscle strength, endurance and mechanical efficiency (11, 12, 23). Intrinsic abnormalities have been described in COPD that are potentially involved in muscle dysfunction. Reduced fiber type I proportions and a I→IIX fiber type shift have been consistently shown (14, 19, 26). In addition, in line with this fiber type redistribution, decreased activities of enzymes involved in oxidative metabolism have been reported (20, 25). Given a potential role for UCP3 in human substrate metabolism (32) and given the fiber type specific expression of UCP3, with higher levels in type II fibers (16), we have recently examined uncoupling protein-3 (UCP3) levels in muscle biopsies from COPD patients and healthy control subjects. In contrast to what could be expected from fiber type distribution, we and others found reduced UCP3 content in COPD instead (13, 30). At present, the exact physiological function of UCP3 is not known, but there is compelling evidence that UCP3 is involved in the mitochondrial fatty acid anion export (34). In situations where fatty acid supply to mitochondria exceeds the oxidizing capacity, fatty acids will accumulate in the matrix where they become deprotonated and can subsequently become peroxidized. UCP3 is suggested to be involved in clearing the matrix from these fatty acid anions (36), thereby protecting the mitochondria against lipid peroxide-induced damage (lipotoxicity). Interestingly, increased levels of intramuscular lipid peroxidation products have indeed been reported for COPD (2, 8). In addition, recent data suggest that the low muscle UCP3 levels in COPD may be linked to disturbed fatty acid metabolism (30).

In accordance with this putative function of UCP3, exercise training improves fat oxidative capacity and therefore allows UCP3 levels to decrease in healthy subjects (31). In COPD patients, exercise training has proven to be beneficial despite severe ventilatory impairment. Some studies showed that improvement in exercise capacity is accompanied by partly restored muscular activities of the oxidative enzymes citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HAD) (24, 28), which is a normal physiological response to endurance training. HAD is an important enzyme involved in the oxidation of fatty acids and therefore of particular interest with respect to UCP3. Since UCP3 is already low in COPD, the question remains whether endurance training in COPD will further reduce UCP3 levels. We therefore examined the effect of an eight-week supervised exercise training intervention on muscular UCP3 levels in this disease. In addition, we monitored changes in lipid peroxidation, and in metabolic profile by activity measurements of glycolytic enzymes and oxidative enzymes, including HAD, as well as myosin heavy chain (MyHC) isoform composition which reflects fiber type distribution.

## **MATERIALS & METHODS**

### **Study population**

A group of 13 patients with moderate to severe airflow obstruction was studied (table 1). All patients had COPD according to ATS guidelines (3) and chronic airflow limitation, defined as measured forced expiratory volume in one second (FEV<sub>1</sub>) less than 70% of reference FEV<sub>1</sub>. Furthermore, patients had irreversible obstructive airway disease (less than 10% improvement of FEV<sub>1</sub> predicted baseline after  $\beta_2$ -agonist inhalation). They were in clinically stable condition and not suffering from a respiratory tract infection or an exacerbation of their

disease at least 4 weeks prior to the study. Only one patient received supplemental oxygen. During rehabilitation patients received maintenance (low dose) respiratory medication that in general consisted of inhaled bronchodilators and inhaled corticosteroids. To confirm previously reported reduced baseline UCP3 levels in COPD, 7 healthy age-matched control subjects were included (table 1). Exclusion criteria were malignancy, cardiac failure, distal arteriopathy, recent surgery, severe endocrine, hepatic or renal disorders and use of anticoagulant medication. Written informed consent was obtained from all subjects and the study was approved by the medical ethical committee of the University Hospital Maastricht (Maastricht, The Netherlands).

### **Intervention**

All patients participated in an eight-week, standardized exercise training program, consisting of a combination of endurance and strength exercise training. The daily program (5 days a week) consisted of 2x 20 min. of submaximal cycle ergometry, 1x 20 min. of treadmill exercise, 1x 30 min. of gymnastics and 1 session of unsupported arm exercise training (consisting of 10 x 1 min. of exercise, each min. followed by 1 min. of rest). A team of experienced physiotherapists tailored each individual training program on the patients' functional impairments in daily living and on the patients' muscular performance.

### **Body composition**

Height was determined to the nearest 0.5 cm with subjects standing barefoot and body weight was assessed to the nearest 0.1 kg while subjects wore light clothing and no shoes. In patients, fat-free mass (FFM) was estimated using single-frequency (50 kHz) bioelectrical impedance analysis (Xitron Technologies Inc., San Diego, CA, USA) while subjects were in a supine position. FFM was calculated using the disease-specific equation (40). In healthy controls,

FFM was determined by dual energy x-ray absorptiometry (DEXA, Lunar Corporation, Madison, WI, USA) (9). Weight parameters were divided by squared height ( $\text{kg}/\text{m}^2$ ), resulting in the body mass index (BMI) and FFM index (FFMI), to adjust for body surface.

### **Pulmonary function tests**

Spirometry was used to determine, amongst others, the  $\text{FEV}_1$ , with the highest value from at least three technically acceptable assessments being used. Diffusion capacity for carbon monoxide was measured by using the single-breath method (Masterlab, Jaeger, Würzburg, Germany). All values obtained were expressed as percentage of the predicted value (29).

Arterial oxygen tension was determined (ABL 330; Radiometer, Copenhagen, Denmark) in a blood sample from the radial artery while breathing room air.

### **Exercise performance**

An incremental bicycle ergometry test was performed on an electromagnetic braked ergometer (Corival 400<sup>®</sup>, Lode, Groningen, The Netherlands) under supervision of a chest physician to investigate maximal leg exercise capacity. After a 2-minute resting period and 1 minute unloaded cycling, power was increased every minute by 10 W. None of the subjects knew the exercise load and all were encouraged to cycle at 60 revs/min until exhaustion. During the exercise test, heart rate, blood pressure and transcutaneous oxygen saturation were monitored. Oxygen consumption ( $\dot{V}\text{O}_2$ ) and carbon dioxide ( $\dot{V}\text{CO}_2$ ) production was measured and calculated from breath by breath analysis using a breathing mask (Oxycon Beta<sup>®</sup>, Jaeger, Würzburg, Germany). Peak workload,  $\dot{V}\text{O}_2$ ,  $\dot{V}\text{CO}_2$  and ventilation ( $\dot{V}\text{E}$ ) were used in the analysis.

### **Collection and processing of muscle tissue**

After at least 3 days without training, postabsorptive muscle biopsies of the lateral part of the *quadriceps femoris* were obtained under local anesthesia by the needle biopsy technique (6). A part of the biopsy ( $\pm 50$  mg) was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further processing. From this, a 5% (w/v) homogenate was prepared by dispersion (Polytron® PT 1600 E, Kinematica AG, Luzern, Swiss) followed by 1 min sonication (Branson 2210, Branson Ultrasonics Corporation, Danbury, USA) of the tissue in SET buffer (250 mM sucrose, 2 mM EDTA, 10 mM TRIS, pH 7.4). Samples were centrifuged (10 min, 10000 G,  $4^{\circ}\text{C}$ ) and the supernatant was used for enzyme activity assays: phosphofructokinase (PFK; EC 2.7.11) (22), 3-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35) (5), citrate synthase (CS; EC 2.3.3.1) (38), and glycogen phosphorylase (GlyP; EC 2.4.1.1) (37), were analyzed spectrophotometrically (Multiskan Spectrum, Thermo Labsystems, Breda, The Netherlands). The remaining pellet was resuspended in three volumes of ice-cold extraction buffer (100 mM  $\text{Na}_4\text{O}_7\text{P}_2 \cdot 10\text{H}_2\text{O}$ , 5 mM EDTA, 1 mM DTT, pH 8.5), incubated on ice for 30 min, and centrifuged (10 min, 10000 G,  $4^{\circ}\text{C}$ ). From this, the supernatant was used for MyHC isoform analysis as described by Talmadge & Roy (41). Gels were run for 22 hrs using a Protean II xi Cell gelectrophoresis system (Biorad, Veenendaal, The Netherlands) at 20 mA with increasing voltage to a maximum of 350 V. About 1.0  $\mu\text{g}$  of protein was loaded per lane. Gels were silver-stained (Silver Stain Plus Kit, Biorad), scanned and photographed with a scanning densitometer (Fluor-S<sup>TM</sup> MultiImager, Biorad), after which bands were quantified using Quantity One software (Biorad). I, IIA and IIX MyHC isoforms were expressed proportionally to each other. The remaining part of the biopsy was placed in a drop of Tissue-tek® (OCT compound) on a piece of cork and frozen in liquid nitrogen. From this, cryosections were collected and homogenized in ice-cold Tris-EDTA buffer at pH 7.4. UCP3 protein content and 4-hydroxy-2-nonenal-(HNE-)protein adducts were determined as described previously (4, 13). In short, from each sample an equal amount of protein was

loaded on a polyacrylamide gel and western blotting was performed using a rabbit polyclonal UCP3 antibody (code 1331; kindly provided by LJ Slieker, Eli Lilly) or a rabbit polyclonal antibody against HNE-protein adducts (Calbiochem, San Diego, CA, USA). Values were normalized using an standard sample to allow for inter-gel comparisons. For UCP3, it was however not possible to use the same standard for the control vs patient comparison and for the patients' before and after training comparison as well, due to limited sample availability. Values were expressed as arbitrary units (AU).

### **Statistics**

Data were analyzed using SPSS (Statistical Package for the Social Sciences, version 11.0 for Windows, SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed with the Mann-Whitney U test. Changes induced by the intervention were analyzed with the Student's *t* test for paired data or, in case of subgroup analyses (with  $N < 10$ ), with the Wilcoxon signed-rank test for paired data. A two-tailed probability value of less than 0.05 was considered statistically significant. Data are presented as the means  $\pm$  SD.

### **RESULTS**

Subject characteristics are shown in table 1. The results of training on the incremental cycle ergometry test in patients are presented in table 2. After the 8 weeks of training, exercise capacity was improved. Both peak  $\dot{V}O_2$  and the peak workload increased significantly (15% and 16%, respectively;  $p \leq 0.015$ ).

Muscular enzyme activities and MyHC isoform composition are presented in table 3. The activities of the two enzymes involved in glycolytic metabolism, GlyP and PFK, were not

different from baseline. In contrast, training induced an increase in oxidative capacity as reflected by the 30% elevated activity of CS.

Compared to the healthy controls, muscular UCP3 protein levels were reduced in COPD patients ( $41.4 \pm 17.3$  vs  $20.7 \pm 17.8$  AU, respectively), as shown in figure 1. In COPD, UCP3 protein level tended to increase after the training intervention (from  $67.7 \pm 41.8$  to  $113.8 \pm 104.2$  AU,  $p=0.062$ ) as shown in figure 2A+B. Figure 2B clearly shows that the training-induced up-regulation of UCP3 only occurred in a subset of the patients. HAD activity did improve in a subset of patients. HAD responders were defined as patients in which the HAD increased with  $>15\%$  (which is the mean increase for the whole group). The 6 HAD responders had an increase in HAD activity (from  $48 \pm 26$  to  $70 \pm 44$  U/mg protein,  $p=0.028$ ), the 7 HAD non-responders had no significant change in HAD activity (from  $54 \pm 34$  to  $48 \pm 26$  U/mg protein). Interestingly, despite the absence of a significant correlation ( $r=-0.43$ ;  $p=0.15$ ) between the changed UCP3 levels and HAD activity, the training-induced change in UCP3 levels was more pronounced in HAD non-responders than in HAD responders ( $87.0 \pm 82.9$  vs  $-1.5 \pm 49.0$  AU,  $p=0.046$ ) as shown in figure 2C. Moreover, in the HAD non-responders UCP3 protein levels significantly increased (from  $80.9 \pm 52.6$  to  $167.9 \pm 109.1$  AU,  $p=0.028$ ), whereas training induced no significant increase in UCP3 levels in the HAD responders (from  $52.2 \pm 18.6$  to  $50.7 \pm 54.0$  AU,  $p=0.753$ ). There were no differences in baseline exercise capacity, body composition or lung function between HAD responders and non-responders. There was no association between baseline UCP3 levels and CS activities, nor between training-induced changes in UCP3 and CS.

Total levels of HNE-conjugated proteins were measured as an index of lipid peroxidation in pre- and post training biopsies from the COPD patients, but there was no significant change

after training (from  $0.50 \pm 0.20$  to  $0.45 \pm 0.15$  AU). There were no correlations between baseline UCP3 and HNE levels, nor between their training induced changes. Also, HNE levels were not different between HAD responders and non-responders.

## **DISCUSSION**

Limb muscles of patients with COPD are characterized by reduced levels of UCP3 compared to healthy controls. The main finding of the current study is that exercise training leads to an upregulation of UCP3 in COPD, which is exactly the opposite to what has been found in healthy subjects. Finally, we show that the UCP3 upregulation predominantly occurs in patients with no improvement in HAD activity. These observations are in line with the putative function of UCP3 as a protector against lipotoxicity.

As previously shown (13), we found that UCP3 levels were ~50% lower in limb muscles of COPD patient compared to healthy age-matched controls. As mentioned earlier, the normal response to exercise training is an increase in oxidative capacity and a down-regulation of UCP3. For the whole group, we indeed found an improved oxidative capacity reflected by an improved CS activity. However, we did not find a further reduction of UCP3 after the 8 week training intervention, but a ~1.7 fold increase instead. Theoretically, the training-induced upregulation of UCP3 in COPD could be the result of changes in fiber type composition and/or mitochondrial content. However, changes in UCP3 levels did not correlate with changes in oxidative capacity, nor with MyHC isoforms. With respect to the fiber type distribution, we found no training-induced changes in the relative composition of the MyHC

isoforms I, IIA or IIX, which is consistent with a previous study that described unaltered fiber type proportions in COPD after a training intervention (42).

How can this opposite response of UCP3 to training in COPD be explained? There now is compelling evidence that UCP3 is not a key regulator of energy metabolism, but that its main function is to protect mitochondria against lipotoxicity: It has been hypothesized that UCP3 serves as a transporter of fatty acid anions out of the mitochondrial matrix, thereby preventing accumulation of these anions which would otherwise be prone to peroxidation by reactive oxygen species, which are also produced in the mitochondrial matrix, leading to lipid peroxidation and subsequently to mitochondrial damage (34). When the influx of fatty acids into the muscle cell exceeds the mitochondrial capacity to oxidize them, fatty acids will accumulate in the cytosol. Besides the active carnitine palmitoyl transferase-1 (CPT1) dependent uptake of fatty acids into the mitochondria, passive fatty acid influx can also occur by a so-called flip-flop mechanism (15). However, fatty acids entering the mitochondrial matrix via the latter route can not be oxidized (as they lack a coenzyme A group), but will be deprotonated and become fatty acid anions. These are unable to flip-flop back over the mitochondrial membrane and need a transporter to be exported. UCP3 has been shown to be able to transport fatty acid anions (39) and in conditions when the fatty acid availability exceeds the oxidative capacity, UCP3 is indeed upregulated (33). In addition, acutely blocking CPT1, which enhances the influx of fatty acids by the flip-flop mechanism, upregulates UCP3 (35). Again, the normal response to training is improved activities of enzymes involved in oxidative metabolism, including that of HAD (10), a marker of fat oxidative capacity. Consistent with the above mentioned function for UCP3, the patients with unimproved HAD activities, referred to as HAD non-responders, showed a marked (2-fold) increase in UCP3 response, whereas UCP3 did not change in the HAD responders. In other

words, the capacity to oxidize fatty acids apparently increased sufficiently in some of the patients, whereas in the other half this was not the case and UCP3 needed to be upregulated. Although the definition of HAD responders and non-responders (more or less improvement compared to the group average) may be somewhat arbitrary, these are interesting observations that deserve more attention in future research.

In line with the above-mentioned mechanism, failure of the “HAD-UCP3 system” would lead to accumulation of fatty acid anions in the inner mitochondrial membrane and/or mitochondrial matrix, and would be prone to lipid peroxidation. Indeed, mice lacking UCP3 were shown to have increased levels of muscular oxidative damage and lipid peroxidation (7). In the current study we measured levels of HNE-conjugated proteins as an index of lipid peroxidation. However, we found no training-induced differences in the patient group. Also, we found no clear association of HNE levels with UCP3 levels or HAD activities. It can be speculated that enhanced lipid peroxidation might be prevented by a sufficient increase of either UCP3 or HAD. On the other hand, it must be stated that the muscle biopsies were obtained at rest and not after acute exercise, a situation in which oxidative stress and lipid peroxidation are much more prominent. It is therefore possible that differences in HNE levels would have been found after acute exercise.

The training-induced upregulation of UCP3 suggests that UCP3 levels are restored roughly to normal levels. The question then remains why UCP3 levels are reduced in COPD patients. This study does not provide the evidence for this, therefore we can only speculate. UCP3 is, at least at the mRNA level, upregulated by circulating fatty acid levels (21). Decreased plasma free fatty acid levels have indeed been reported in COPD (18). The effect of fatty acids on UCP3 expression probably involves the peroxisome proliferator-activated receptors (PPARs),

as the promoter of UCP3 has been shown to contain a PPAR responsive element (1). It could be speculated that PPAR activity is reduced in COPD, although at present the PPARs have not been studied in COPD for that purpose. Interestingly, PPARs may be down-regulated by hypoxia (27). In addition, Zhou et al showed that acute hypoxia upregulated UCP3, but that prolonged exposure to hypoxia lead to a reduction of UCP3 in rat muscle (43). It is therefore tempting to hypothesize that chronic hypoxia is involved in reduced UCP3 levels in COPD. Although we did not evaluate daily activity patterns in this study, it can be assumed that COPD patients are less physically active than healthy elderly and that muscle disuse is also involved in reduced UCP3 levels. This is however unlikely, because the effect of inactivity has been studied previously in tetraplegic subjects, a condition characterized by profound muscle disuse, and UCP3 was found to be upregulated in this model (17). Hence, more research is required to determine which factors are indeed involved in the low UCP3 contents in COPD and what the underlying molecular mechanisms are.

In summary, UCP3 levels are reduced in untrained sedentary patients suffering from COPD. This can be restored by an exercise training intervention, which has been shown to improve oxidative capacity as well. The fact that the upregulation of UCP3 in response to training does not occur in patients with a sufficient increase in HAD activity is entirely in line with the accumulating evidence that physiological function of UCP3 is to protect the mitochondria against lipotoxicity. More research is required to establish if reduced UCP3 levels in COPD reflect a pathophysiological state and whether UCP3 is a potential target for therapeutic intervention.

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## **DISCLOSURES**

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## LEGENDS

Figure 1: Box plot showing the Western Blot results for UCP3 protein levels in COPD patients compared to controls. AU = arbitrary units; \* = statistically significant difference.

Figure 2. A) Western Blot results for UCP3 from all COPD patient muscle biopsies. Baseline and post-training samples are labeled t0 and t8 respectively. On every gel a standard sample was included (S), to correct for differences between gels/blots. B) Individual pre- and post-training UCP3 levels. Horizontal bars indicate means; AU = arbitrary units. C) Box plot showing the change in UCP3 level in HAD responders (subjects with >15% increase in HAD activity after training) and HAD non-responders. \* = statistically significant difference.

Figure 3. A) Representative Western Blot results for HNE-protein adducts. Optical densities of bands ranging from ~10 to ~100 kDa were measured and total HNE-protein adduct levels were calculated. On every gel a standard sample was included (S), to correct for differences between gels/blots. HNE, 4-hydroxy-2-nonenal.

**TABLES**

Table 1: Subject characteristics

	COPD	Controls
N (Male/Female)	13 (6/7)	7 (4/3)
Age (yr)	61 ± 12	64 ± 3
Smokers / ex-smokers / never smoked	1 / 12 / 0	1 / 4 / 2
Height (m)	1.67 ± 0.09	1.64 ± 0.08
Weight (kg)	63 ± 11	72 ± 9
BMI (kg/m <sup>2</sup> )	22.7 ± 1.1	26.7 ± 4
FFM (kg)	44 ± 6.9	49 ± 8.2
FFMI (kg/m <sup>2</sup> )	15.9 ± 2.2	18.0 ± 2.3 *
PaO <sub>2</sub> (kPa)	9.0 ± 1.2	-
PaCO <sub>2</sub> (kPa)	5.4 ± 0.7	-
FEV <sub>1</sub> (%pred)	37.1 ± 11.8	119 ± 22 †
DL <sub>CO</sub> (%pred)	47.0 ± 23.5	-
FVC (%pred)	75 ± 11	123 ± 21 †

Values are mean ± SD. BMI, body mass index; FFM, fat-free mass; FFMI, fat-free mass index; PaO<sub>2</sub>, arterial oxygen pressure; PaCO<sub>2</sub>, arterial carbon dioxide pressure; FEV<sub>1</sub>, forced expiratory volume in one second; DL<sub>CO</sub>, diffusion capacity for carbon monoxide; FVC, forced vital capacity; %pred, percent of predicted. Significance of difference between groups: \* p<0.05; † p<0.001.

Table 2: Incremental cycle ergometry test

	Baseline	After Training	
Peak Load (W)	55.7 ± 27.5	67.1 ± 32.8	*
Peak $\dot{V}O_2$ (ml/min)	902 ± 327	1045 ± 316	*
Peak $\dot{V}CO_2$ (ml/min)	896 ± 485	997 ± 373	
Peak RQ	0.96 ± 0.16	0.94 ± 0.12	
Peak $\dot{V}E$ (l/min)	35.8 ± 14.1	41.5 ± 12.9	*

Values are mean ± SD. \* indicates a significant change ( $p < 0.05$ ). RQ, respiratory quotient;  $\dot{V}E$ , ventilation.

Table 3: Muscular changes after intervention

	Baseline	After Intervention	
MyHC I (%)	24.5 ± 7.2	22.2 ± 5.8	
MyHC IIA (%)	47.4 ± 11.2	46.3 ± 7.1	
MyHC IIX (%)	28.1 ± 10.3	31.5 ± 8.7	
GlyP (U/mg protein)	185 ± 107	192 ± 117	
PFK (U/mg protein)	674 ± 372	641 ± 327	
CS (U/mg protein)	32 ± 22	42 ± 28	*
HAD (U/mg protein)	51 ± 32	58 ± 36	

Values are mean ± SD. \* indicates a significant change ( $p < 0.05$ ). MyHC, myosin heavy chain; GlyP, glycogen phosphorylase; PFK, phosphofructokinase; CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase.

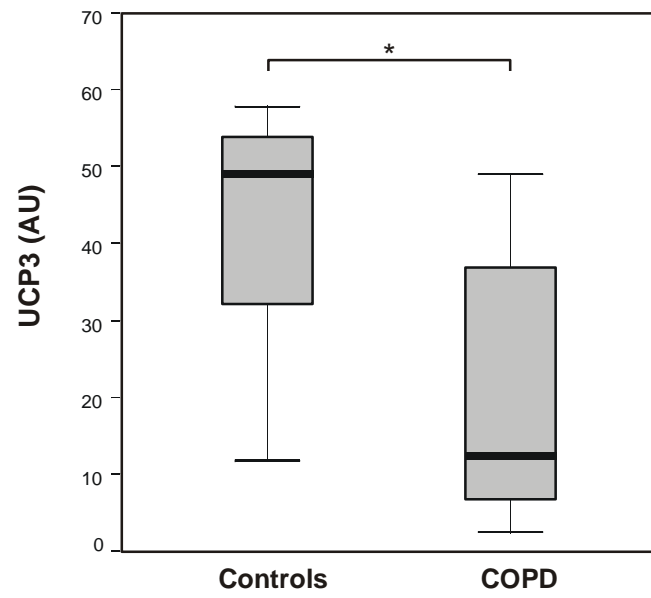
**FIGURES**

Figure 1

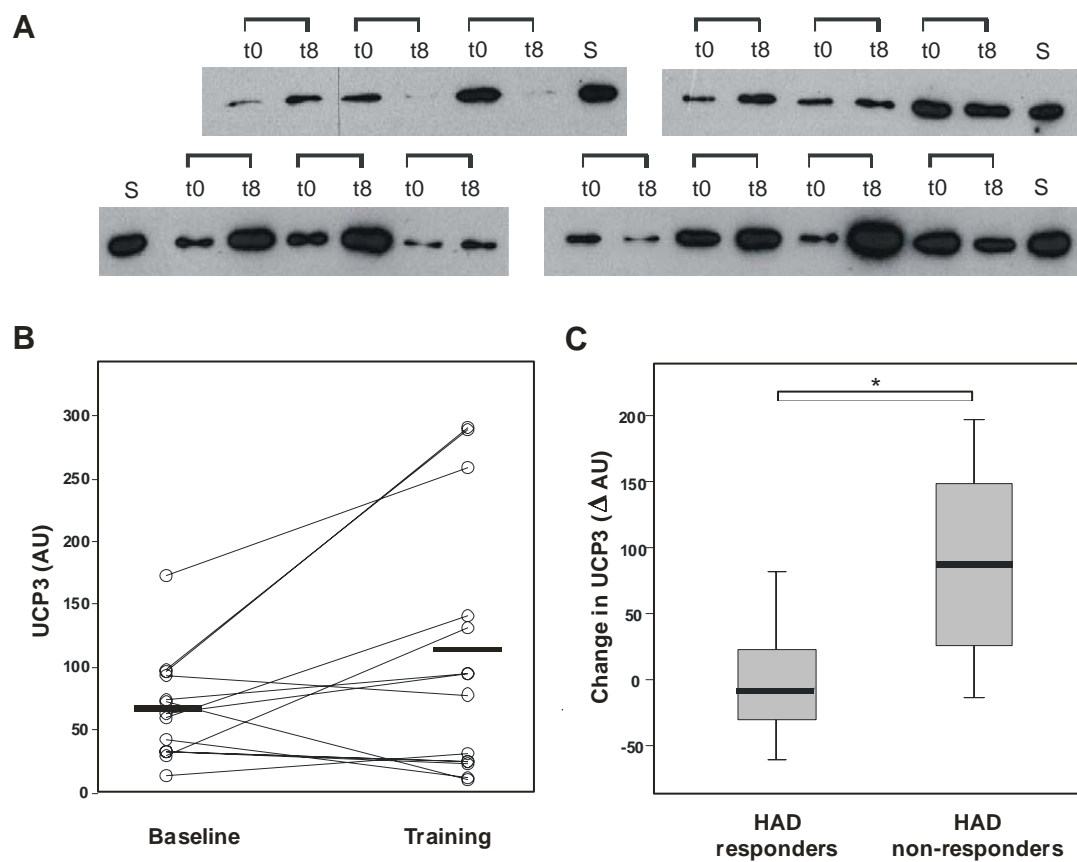


Figure 2

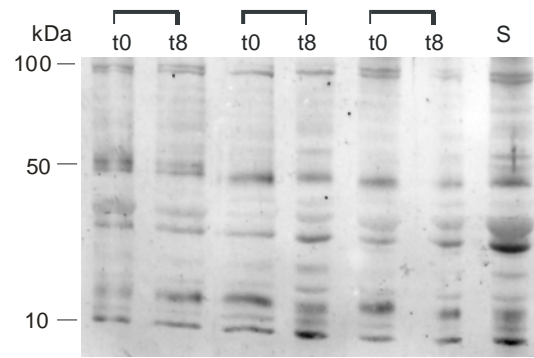


Figure 3.